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Influence of Pilin Glycosylation on *Pseudomonas aeruginosa* 1244 Pilus Function

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The opportunistic pathogen *Pseudomonas aeruginosa* is a leading cause of nosocomial pneumonia. Among its virulence factors, the type IV pili of *P. aeruginosa* strain 1244 contain a covalently linked, three-sugar glycan of previously unknown significance. The work described in this paper was carried out to determine the influence of the *P. aeruginosa* 1244 pilin glycan on pilus function, as well as a possible role in pathogenesis. To accomplish this, a deletion was introduced into the *pilO* gene of this organism. The isogenic knockout strain produced, 1244G7, was unable to glycosylate pilin but could produce pili normal in appearance and quantity. In addition, this strain had somewhat reduced twitching motility, was sensitive to pilus-specific bacteriophages, and could form a normal biofilm. Analysis of whole cells and isolated pili from wild-type *P. aeruginosa* strain 1244 by transmission electron microscopy with a glycan-specific immunogold label showed that this saccharide was distributed evenly over the fiber surface. The presence of the pilin glycan reduced the hydrophobicity of purified pili as well as whole cells. With regard to pathogenicity, *P. aeruginosa* strains producing glycosylated pili were commonly found among clinical isolates and particularly among those strains isolated from sputum. Competition index analysis using a mouse respiratory model comparing strains 1244 and 1244G7 indicated that the presence of the pilin glycan allowed for significantly greater survival in the lung environment. These results collectively suggest that the pilin glycan is a significant virulence factor and may aid in the establishment of infection.

Pseudomonas aeruginosa is a gram-negative pathogen capable of causing severe infections in individuals with compromised defense mechanisms (5). This organism elaborates multiple virulence factors, including type IV pili, protein fibers extending from the cell's pole (45). These pili contribute to the disease process by promoting colonization through adhesion and by mediating a method of surface translocation called twitching (30). They are composed primarily of a monomeric subunit called pilin, a protein derived from a precursor by cleavage of its six amino-terminal residues and the N-methylation of the newly revealed phenylalanine at this terminus (46). Pilin of *P. aeruginosa* 1244 is further modified by glycosylation (7).

Until recently, common knowledge held that prokaryotes did not glycosylate proteins. This was thought to be so even though copious evidence existed that glycans were found covalently attached to archeal and eubacterial S-layer proteins (25, 26, 31, 51). More recent work has led to the appreciation that prokaryotic protein glycosylation is widespread, with a large proportion being associated with the cell surface of pathogens. Among the gram-positive bacteria, several examples of *Streptococcus* species important in periodontal disease have been shown to produce glycosylated proteins (3). In addition, *Mycobacterium tuberculosis* envelope proteins along with a potential adhesin of this organism have been proven to be glycosylated (16, 19), and a major component of the exosporium layer of the *Bacillus anthracis* endospore is a glycoprotein (47). Numerous examples of surface protein glycosylation among the gram negatives also exist, including adhesins of *Escherichia coli* (4, 29), *Chlamydia trachomatis* (23), and *Haemophilus influenzae* (18). Protein glycosylation in *Campylobacter* species has been well studied, where two glycosylation systems have been demonstrated, one targeting secreted proteins and the other limited to flagellin glycosylation (48). Glycosylation of flagellin subunits has been examined also in *Pseudomonas aeruginosa* (36) and in *Helicobacter pylori* (37). Pilin glycosylation also occurs in *Neisseria meningitidis* (44).

Structural analysis of the *P. aeruginosa* 1244 pilin glycan indicates that this moiety is a trisaccharide identical with the lipopolysaccharide O-antigen repeating unit of this strain (8). Each pilin monomer possesses a single glycan, with no evidence of either nonglycosylated subunits or other alternate glycoforms (8). Analysis of a purified aminoglycan produced by total proteolysis of pure glycosylated pilin showed the presence of a serine, indicating that the glycan is covalently bound via the β-carbon of this residue (8). Site-directed mutagenesis of the gene coding for pilin (*pilA*) and sequence analysis of pilin endopeptidase fragments revealed that the serine to which the glycan is attached is the carboxy-terminal residue of this protein (11). The structure of the strain 1244 pilin glycan suggests that it is a product of the O-antigen biosynthetic pathway. In support of this idea, it has been shown that 1244 mutant strains defective in specific steps in O-antigen biosynthesis are unable

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to make glycosylated pili (15). Confirmation of this suggestion came with the finding that cloned O-antigen biosynthesis gene clusters coding for heterologous antigen could be expressed in *P. aeruginosa* 1244 producing pilin glycosylated with the heterologous saccharide (15).

pilO, a gene required for pilin glycosylation (7), is predicted to code for a hydrophobic and basic protein with a molecular weight of 50,682 and exists as part of an operon which includes *pilA*, the gene encoding pilin. PilO is the presumed glycosyltransferase responsible for attachment of the O-antigen repeating unit to pilin. Expression of a cloned 1244 *pilAO* operon in a heterologous *P. aeruginosa* strain, which normally produces nonglycosylated pilin, results in the production of glycosylated pilin (15). Furthermore, only 1244 pilin lacking glycan was made when the strain 1244 *pilA* gene alone was expressed in this genetic background. These results suggest that PilO is the only glycosylation factor required that is not part of either the pilin or the O-antigen pathways.

The inner core of the type IV pilus fiber is composed of the pilin hydrophobic α-helical tail, while the globular head region of this stickpin-shaped protein makes up the fiber surface structure (13). The residue to which the strain 1244 glycan is covalently attached is immediately adjacent to the type IV pilin-specific disulfide loop (DSL) region, a structure important in host glycolipid-specific binding (40). Lee et al. (27) showed that the DSL is at least partially buried in the outer protein layer of the pilus shaft and is only surface exposed at the fiber tip. These results suggest that the pilin glycan might also not be present at the fiber surface or be surface expressed only at the pilus tip. Occurrence of this negatively charged and hydrophilic structure at the pilus surface would be expected to have a significant influence on the interaction of the fiber with its environment, a situation that would likely influence pilus function. Work presented in this paper employing immunogold labeling has shown that the glycan, unlike the DSL, is surface located over the entire pilus. We also show, employing a *P. aeruginosa* 1244 isogenic knockout mutant unable to glycosylate pilin, that the presence of the glycan strongly modulates pilus surface hydrophobicity. Evidence that *P. aeruginosa* strains producing glycosylated pili are commonly found among clinical isolates is presented. In addition, we show that pilin glycosylation increases colonization as determined by the mouse acute pneumonia model.

MATERIALS AND METHODS

Organisms and growth conditions. The bacterial strains, viruses, and plasmids used are listed in Table 1. All bacterial strains were routinely grown aerobically at 37°C. Broth cultures were grown on a rotary shaker at 275 rpm. LB plates or broth were used for routine culture growth. The following selective agents were employed in media at the indicated concentrations: ampicillin at 100 μg/ml for *Escherichia coli*; carbenicillin (Cb) at 250 μg/ml for *Pseudomonas aeruginosa* 1244; gentamicin (Gm) at 15 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa*; tetracycline at 15 μg/ml for *E. coli* and 50 μg/ml for *P. aeruginosa*. Isopropyl-β-D-thiogalactopyranoside (IPTG) was employed at a concentration of 5 mM.

Generation of a strain 1244 PilO null mutant by allelic replacement. The BamHI/EcoRI fragment of pPAC202 was ligated with pNOT322 cut with these same enzymes, producing pGIL100. An XhoI site of this construct was removed by digestion with EcoRI and XbaI followed by Klenow polishing and religation, producing pGIL101. A DNA fragment containing the gentamicin resistance gene of pPC110 was amplified using the following oligonucleotide primers: (i) NILIP15 (5′-GCGCGCCTCGAGCAGCTATGACCATGATTACGAATTCCCC-3′), which contained an XhoI site, and (ii) NILIP 16 (5′-GCCCCCGCTAGCCGTTGTAAAACGACGGCCAGTGAATTCCCC-3′), which contained a NheI site. The DNA produced was digested with NheI and XhoI and ligated with pGIL101 digested with the same enzymes. This produced a construct, pGIL200, that contained a 1,268-bp deletion in the *pilO* gene. The NheI/XhoI fragment of pGIL200 containing the gentamicin resistance gene was ligated with pPAC124, a plasmid containing the *pilAO* operon, cut with the same enzymes producing pPUC46Gm. The PstI/HindIII fragment of this construct, which contained the mutated *pilO* gene, was ligated with pEX18Tc cut with these same enzymes, producing pGIL300. pGIL300 was moved into *P. aeruginosa* 1244 by triparental mating. Tetracycline- and gentamicin-resistant clones provided evidence that the plasmid had integrated into the bacterial chromosome. Continued subculturing of these clones on selective medium containing gentamicin and 5% sucrose gave rise to colonies that were tetracycline sensitive, suggesting the loss of the functional *pilO* gene. This was confirmed by Southern blot analysis using strain 1244 *pilA* DNA as a probe. This mutant was referred to as strain 1244G7.

Construction of pMBT100. The XbaI/NheI fragment of pPAC124, which contained the *P. aeruginosa* 1244 *pilA* gene, was removed and the vector was religated. The BamHI/HindIII fragment of this plasmid was ligated with pT7-7 (49) cut with the same enzymes. This construct was digested with EcoRI and BamHI, the overhangs were filled in using DNA polymerase, and the blunt ends were religated. The XbaI/HindIII fragment from this plasmid containing the *pilO* gene was ligated with pUC18 digested with the same enzymes. The EcoRI/HindIII fragment from this construct was ligated with pMMB66EH (17) digested with the same enzymes. This construct was referred to as pMBT100 and contained the strain 1244 *pilO* gene under control of a *tac* promoter.

Isolation and purification of pili. Glycosylated strain 1244 pili were harvested as previously described (7). Nonglycosylated strain 1244 pili were produced from strain 1244G7 using a procedure described by Silipigni-Fusco (42). Here, CAYE solid medium, which was composed of 0.75% Casamino Acids, 0.15% yeast extract, and 2% agar, was used for cell growth. Cultures were grown in foil-covered 68- by 28- by 3-cm metal pans containing 500 ml of CAYE medium for 14 h at 37°C. Cells from each pan were resuspended with 50 ml of 4.0 mM sodium potassium phosphate, pH 7.0, and stirred vigorously for 30 min at room temperature. Cells were removed by centrifugation at 16,000 × *g* for 30 min at 4°C, leaving the pili suspended in the supernatant fluid. Both glycosylated and nonglycosylated pili were purified by repeated precipitation in the presence of 3% polyethylene glycol and 0.5 M NaCl (42).

In order to remove polyethylene glycol, which interfered with mass analysis using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis, 2 to 5 mg of pili was first dialyzed against 6 liters of deionized water containing 0.025% sodium azide and then bound to a Mono-Q column equilibrated with 0.02 M Tris-HCl, pH 7.3. Pili were eluted with a 1% solution of sodium dodecyl sulfate (SDS) and dialyzed as described above. This material was lyophilized and resuspended with 0.5 ml of 67% ethanol. After overnight incubation at 4°C, the material was centrifuged and the pellet was lyophilized.

Transmission immuno-electron microscopy. Cells grown for 14 h at 37°C on CAYE agar were gently suspended in phosphate-buffered saline (PBS) and applied to a formvar-coated copper grid. This material was fixed at room temperature for 5 min with 2% paraformaldehyde and 0.01% glutaraldehyde in PBS. The grid was then subjected at room temperature to three 5-min PBS washes, three 5-min treatments with 0.5% bovine serum albumin (BSA) in PBS solution, and a 30-min incubation in 5% normal goat serum in the BSA solution, followed by three more 5-min washes in the BSA solution. The primary antibody employed, monoclonal 11.14, recognizes both the O-antigen and the pilus glycan of *P. aeruginosa* 1244 (8). The treated grid was incubated for 60 min at room temperature with this antibody diluted 10^{-3} in PBS, followed by three 5-min washes with the BSA solution. The sample was then incubated with secondary antibody, goat anti-mouse immunoglobulin G labeled with 5-nm gold particles (Amersham Biosciences, Piscataway, N.J.) for 60 min at room temperature. The grid was washed with the BSA solution three times for 5 min each and PBS three times for 5 min each, after which the entire grid was fixed in 2.5% glutaraldehyde for 5 min. After three 5-min PBS washes and a 1-min distilled water wash, 2% phosphotungstic acid, pH 6, was briefly applied to the grid and excess solution was wicked away with filter paper, after which it was allowed to dry. Images were taken using a JEM 1210 computer-controlled high-contrast 120-kV transmission electron microscope.

Hydrophobicity assays. To measure pilus hydrophobicity, approximately 2 mg of purified pili from strains 1244, 1244G7, and PA103 was resuspended in 0.5 ml of 10 mM Tris-HCl, pH 8.0, in a microcentrifuge tube. Enzyme-grade ammonium sulfate was added to the desired percent saturation, after which the sample was incubated at room temperature with shaking for 30 min after the salt had gone into solution. The tubes were centrifuged at 14,000 × *g* for 10 min, and supernatant fluid was transferred to a fresh tube. This procedure was repeated to

TABLE 1. Strains, plasmids, and bacteriophages used in this study

Strain plasmid, or virus	Description	Source or reference
P. aeruginosa strains		
1244	Wild-type strain, group I pili	34
1244G7	PilO-negative mutant	This study
1244wbpL	O-antigen-negative mutant	15
1244N3	*rpoN*	33
9D2	Wild-type strain, group I pili	9
577B	Wild-type strain, group I pili	9
653A	Wild-type strain, group I pili	C. C. Brinton, University of Pittsburgh
134VA	Wild-type strain, group I pili	C. C. Brinton, University of Pittsburgh
PA103	Wild-type strain, group II pili	9
PAK	Wild-type strain, group II pili	9
T2A	Wild-type strain, group II pili	9
Isolate collection	Clinical isolates	S. D. Kominos, Mercy Hospital, Pittsburgh
Plasmids		
pC110	Ap^r Gm^r, pUC7 derivative	50
pEX18Tc	Tc^r *oriT SacB*, allelic replacement vector	39
pGIL100	Ap^r, pNOT322 containing *pilAO* operon	This study
pGIL101	Ap^r, pGIL100, EcoRI/XbaI fragment removed	This study
pGIL200	Ap^r, Gm^r, 1.3-kb replacement of *pilO* DNA	This study
pGIL300	Tc^r, *sacB*, PstI/HindIII fragment from pPAC124GM	This study
pMBT100	Ap^r, 1.2-kb insert containing 1244 *pilO* under tac promoter in pMMB66EH	This study
pMMB66EH	Ap^r, broad-host-range vector	17
pNOT332	Ap^r Tc^r, cloning vector	38
pPAC24	Ap^r, 0.8-kb insert containing strain 1244 *pilA* under tac promoter in pMMB66EH	7
pPAC46	Ap^r, 2.3-kb insert containing strain 1244 *pilAO* under tac promoter in pMMB66EH	7
pPAC124	Ap^r, 2.3-kb insert containing strain 1244 *pilAO* under tac promoter in pUC18	7
pPAC202	Tc^r, subclone containing strain 1244 *pilAO* operon	10
pUC46GM	Ap^r Gm^r, pPAC124 with 1.3-kb replacement of *pilO* DNA	This study
pUCP19*exoUspcU*	Ap^r, pUC19-based plasmid carrying *exoU* and *spcU*	1
pT7-7	Ap^r, cloning vector containing T7 RNAP promoter	49
Viruses		
PE69, PO4, M6,	*P. aeruginosa* pilus-specific RNA bacteriophages	6
F116	*P. aeruginosa* pilus-specific RNA bacteriophages	22

produce the desired fractionation range. Precipitates were resuspended in deionized water and tested for protein concentration using the BCA assay (Pierce). To test cell hydrophobicity, strains were grown on CAYE agar plates for 14 h at 37°C. Growth formed on individual plates was gently resuspended with 5 ml of CAYE broth. Growth was estimated by determining optical density at 650 nm, and cultures were normalized to give 2×10^{10} cells per ml. A 15-μl aliquot of suspended cells was mixed on a microscope slide with an equal volume of 0.5, 1.0, or 2.0 M ammonium sulfate and incubated for 25 min at room temperature. The samples were examined at timed intervals for agglutination using a dissection microscope.

SDS-PAGE, isoelectric focusing, and immunoblot analysis. Pilin samples were analyzed electrophoretically using 15.0% T, Tris-glycine SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Pili samples to be subjected to isoelectric focusing were treated with 2% β-octylglucoside for 15 min at room temperature. This material was separated in a pH gradient from 3.5 to 9.0 using a Pharmacia PhastSystem apparatus in which the gels had been equilibrated with 1.0% β-octylglucoside. Pilin separated by SDS-PAGE was transferred to nitrocellulose membrane by electroblotting, whereas diffusion blotting was used for transfer of pilin separated by isoelectric focusing. For immunoblot analysis, blocking was carried out as previously described (10) and either monoclonal antibody 11.14 (8), 6.45 (41), 5.44 (41), or 2.97 (35) was used as probe. Screening clinical isolates by Western blotting employed an anti-1244 pilin polyclonal preparation (11). Reaction for these assays was determined using an alkaline phosphatase-labeled secondary antibody as has been described previously (10). For pilin quantitation using Western blotting, a fluorescein isothiocyanate (FITC)-labeled secondary antibody was employed.

Analytical methods. Mark Bier of the Mellon Institute Center for Molecular Analysis, Carnegie Mellon University, carried out pilin mass determinations by MALDI-TOF using a PerSeptive Biosystems Voyager STR with DE and a high *m/z* detector. Detection and quantitation of Western blots using FITC-labeled secondary antibody were carried out using a Molecular Dynamics model 595 Fluorimager equipped with ImageQuant software.

Pilus functionality assays. Twitching motility was determined using the protocol described previously (8). The protocol of O'Toole and Kolter (32) was employed to determine biofilm formation. Bacteriophage sensitivity was tested using the cross-streak method described previously (10).

Clinical isolate screening. *P. aeruginosa* strains tested for pilus type were obtained from Spyros Kominos, Mercy Hospital, Pittsburgh, Pa. These isolates were grown overnight at 37°C on LB agar plates. Whole-cell extracts were separated by PAGE and analyzed by Western blotting using polyclonal antibodies raised by immunization of mice with pure strain 1244 pili.

Strain competition assay. The acute pneumonia model was carried out using 6- to 8-week-old female BALB/c mice (Hilltop Lab Animals, Scottdale, PA). Animal experiments complied with institutional and federal guidelines regarding the use of animals in research. Prior to challenge, mice were anesthetized with intraperitoneal injections of 0.25 ml of freshly prepared and filter-sterilized ketamine (6.7 mg/ml) and xylazine (1.3 mg/ml) in 0.9% saline. Challenge cells were grown for approximately 14 h at 37°C on tryptic soy agar (TSA) plates. These cells were gently resuspended with sterile saline and diluted to the approximate desired inoculum level (also with sterile saline) by absorbance at 650 nm. A 10-μl aliquot of the cell suspension diluted with sterile saline was applied to each nostril (20 μl per mouse), after which the animals were monitored (for

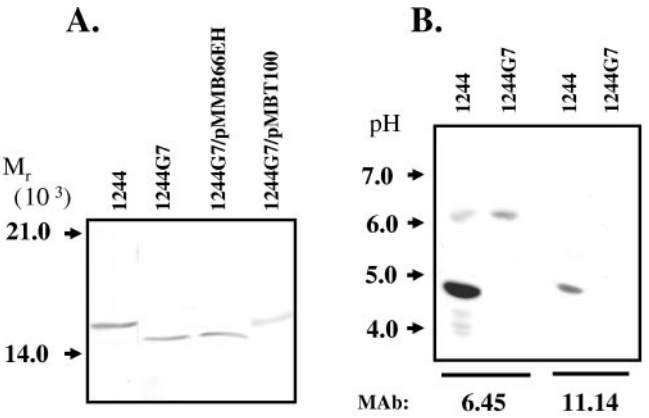

FIG. 1. Immunoblots of pilin from *P. aeruginosa* 1244 and 1244G7. (A) Western blot of cell extracts prepared from overnight plate cultures using the pilin protein-specific monoclonal 6.45. *P. aeruginosa* 1244G7/pMMB66EH and *P. aeruginosa* 1244G7/pMBT100 were grown in the presence of Cb and IPTG. Arrows indicate the position of molecular weight standards. (B) Electrofocusing immunoblot of purified pilin. Arrows indicate the focusing gel pH gradient.

a period of 4 days) for morbidity and mortality. Actual CFU of the inocula were determined by plating on TSA plates. Using this procedure, the 50% lethal dose (LD_{50}) of strain 1244 had previously been determined to be 6.3×10^6 CFU (results not shown). It was deemed desirable to reduce this value in order to minimize any nonspecific inflammation effect caused by the challenge dose. Previous work has shown that the presence of *exoU* can be correlated with increased pathogenicity in the respiratory model (1). We determined by PCR, using primers specific for the ExoU-positive strain *P. aeruginosa* PA103, that strain 1244 lacks *exoU* (results not shown). The constitutive expression of *exoU* from pUC19*exoUspcU* in strain 1244 resulted in ExoU expression as determined by PCR, using the above-mentioned primers, and reduced the LD_{50} of strain 1244 to 1.8×10^5. The acute pneumonia model was employed for the competition assay, using the procedure for anesthetization and challenge described above. Here, a suspension containing known amounts of *P. aeruginosa* strains 1244 and 1244G7 (totaling one strain 1244 LD_{50}) was administered to each of six mice. These animals were sacrificed after 12 h, and lung, liver, and spleen were removed, weighed, and homogenized in 1 ml sterile saline. In order to determine the total cell count and to differentiate between strains 1244 and 1244G7, the homogenate was diluted in sterile saline and plated on TSA-Cb^{250} and TSA-Cb^{250} Gm^{100}.

RESULTS

Mutant isolation and characterization. A *P. aeruginosa* 1244 mutant deficient in the ability to glycosylate pilin was produced in order to study the influence of the pilin glycan on pilus structure and function. This strain, 1244G7, had a 1,268-bp segment of *pilO*, a gene which had previously been shown to be required for pilin glycosylation (7), replaced with a gentamicin resistance cartridge. A Western blot assay using a 1244 pilin-specific monoclonal antibody as probe showed that pilin produced by strain 1244G7 had a lower apparent molecular weight than that produced by the wild-type strain (Fig. 1A). MALDI-TOF analysis (Table 2) of purified mutant pilin indicated that this protein had a mass of 15,650 (±30), which agrees with the value predicted by gene structure (7). Wild-type 1244 pilin, as described previously (8), has a mass of 16,307 (±25). The difference between the mutant and wild-type pilins is approximately the value determined for the strain 1244 glycan structure (8).

For confirmation of these results, pili produced by strain 1244G7 were depolymerized by detergent treatment and the subunits produced were subjected to isoelectric focusing, blotted to nitrocellulose paper, and probed with pilin- or glycan-specific monoclonal antibodies. The results (Fig. 1B) showed that while, as described previously (7), pilin produced by the parent strain focused at pH 4.75, pilin produced by 1244G7 focused at approximately 6.25, a value consistent with a non-glycosylated subunit. Further, while glycosylated 1244 pilin was recognized by a glycan-specific monoclonal antibody, pilin from the mutant did not react. To see if the introduction of a functional *pilO* gene complemented this defect, pMBT100, a plasmid that contained a functional copy of the *pilO* gene under control of an inducible promoter, was introduced into strain 1244G7. While a vector control produced nonglycosylated pilin, the inclusion of *pilO* restored the ability to produce glycosylated pilin (Fig. 1A). Overall, these results show that 1244G7 is able to produce only nonglycosylated pilin.

TABLE 2. Mass determination of *P. aeruginosa* pilins

Pilin source	Predicted mass (Da)[a]	Measured mass (Da)
1244	15,648	16,307 (±25)[b]
1244G7	15,648	15,650 (±30)
PA103	15,169	15,192 (±30)
PAK	15,018	15,020 (±30)
T2A	14,727	14,760 (±30)

[a] Value predicted by gene structure and corrected for N-methylation and loss of cysteine protons.
[b] As determined previously (8).

In order to determine whether the absence of the glycan resulted in a reduction of piliation, strain 1244 and 1244G7 plate-grown cells were subjected to a shearing treatment that was followed by isolation of the pili produced. Sheared pili were analyzed by Western blotting using a pilin protein-specific primary antibody and a secondary antibody labeled with a fluorescent dye. Quantitation of fluorescence showed that the mutant produced approximately 95% of the amount of extracellular pilin made by the wild-type strain. These results indicated that the presence of the pilin glycan had little influence on the degree of piliation. A standard twitching assay indicated that 1244G7 cells produced twitch zones that had an approximately 33% smaller diameter than wild-type cells (results not shown). A quantitative assay showed that both strains produced approximately the same amount of biofilm. Finally, using a cross-streak assay, it was determined that strain 1244 was sensitive to pilus phages PE69 and PO4, but not F116 or M6. A mutant strain, 1244.47, which was unable to produce pilin, was shown to be resistant to PE69 and PO4, confirming that these viruses were pilus specific (results not shown). Strain 1244G7 was found to also be sensitive to these phages, indicating that the absence of pilin glycan did not interfere with phage attachment to the pilus. These results suggest that pilin glycosylation does not strongly influence basic pilus function. In addition, growth curves of strains 1244 and 1244G7 are identical (results not shown), indicating that neither the loss of the *pilO* gene nor the presence of the Gm cartridge makes the mutant less physiologically fit.

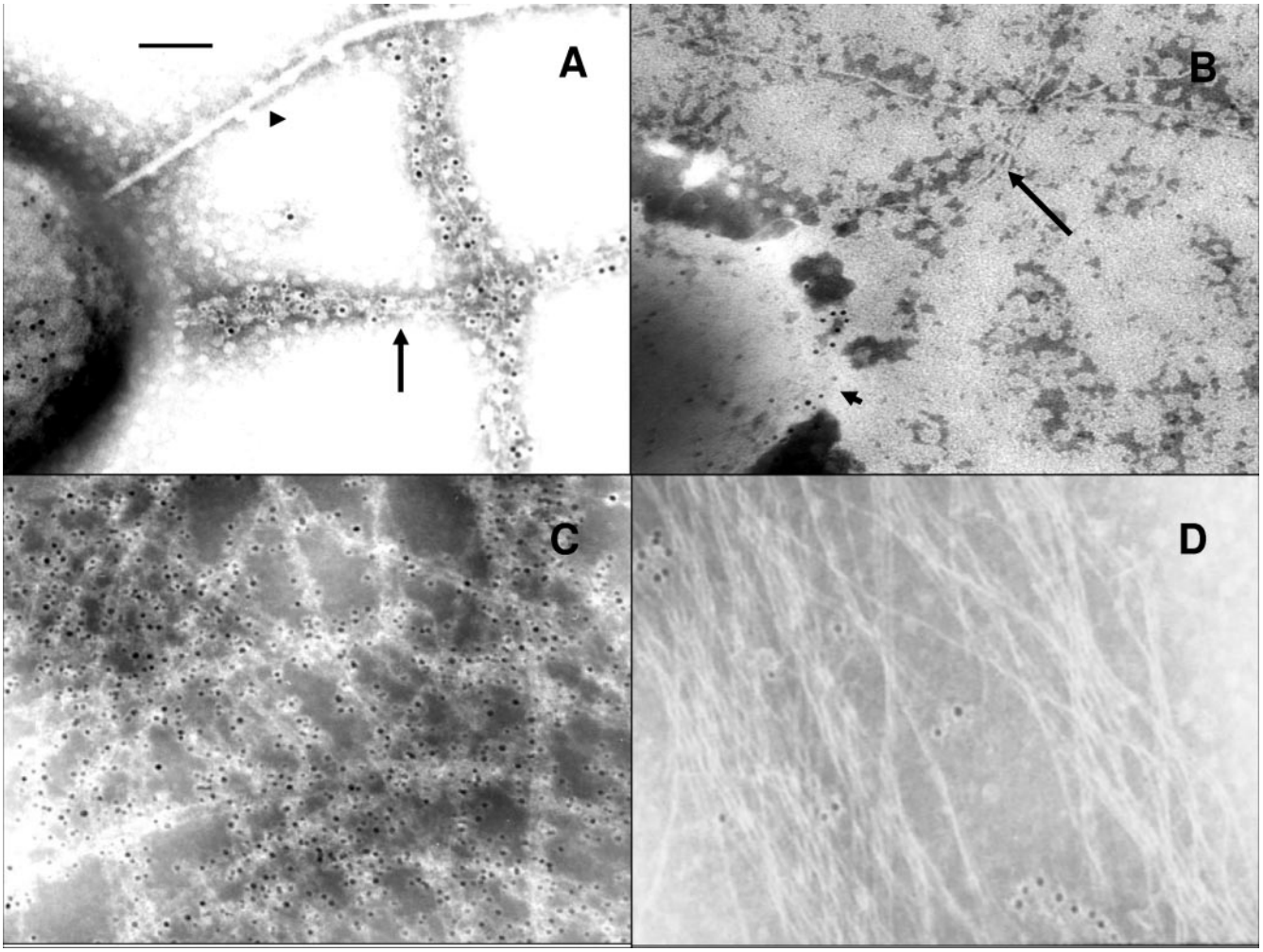

FIG. 2. Transmission electron micrographs of cell-associated and purified pili of *P. aeruginosa* 1244 and *P. aeruginosa* 1244G7. (A) Strain 1244 cells probed with monoclonal 11.14 and gold-labeled secondary antibody. The arrow indicates pili, while the arrowhead marks the flagellum. (B) Strain 1244G7 cells probed with monoclonal 11.14 and gold-labeled secondary antibody. The arrow indicates pili, while the arrowhead marks the cell. (C) Strain 1244 pili probed with monoclonal 11.14 and gold-labeled secondary antibody. (D) Strain 1244G7 pili probed with monoclonal 11.14 and gold-labeled secondary antibody. Bar, 100 nm.

Pilus glycan location and distribution. In order to assess the potential influence of the pilin glycan on the interaction between the pilus and its immediate environment, it was first necessary to show that the glycan occupies a position for such contact. We have previously observed that the pilin glycan is covalently attached to the carboxy-terminal residue of this protein (11). This places it immediately adjacent to the type IV pilin-specific DSL region. Previous studies have determined that the pilin DSL of *P. aeruginosa* PAK is only surface located at the pilus tip and that it is in at least a partially buried form on the fiber shaft (28). With this in mind it was necessary to determine if the glycan was present at the fiber surface and, if so, whether it was distributed evenly over the surface or limited to a particular portion of the pilus.

To do this, cell-associated pili and purified pili of strain 1244 were examined using transmission electron microscopy. Samples, fixed on a coated copper grid, were treated with a monoclonal antibody that, while specific for 1244 O-antigen, also recognized the pilin glycan (8). A gold-labeled secondary antibody allowed visualization of the site of monoclonal recognition. Figure 2A shows label associated with the pilus, indicating that the glycan is present at the fiber surface. Further, the absence of reaction with the flagellum indicates that the pilus-associated reaction was not due to LPS contamination. The presence of label on the cell surface suggested that the probe recognized lipopolysaccharide-bound O-antigen or glycosylated pilin subunits associated with the outer membrane. Examination of 1244G7 cells using this same protocol revealed that this organism produced pili that were indistinguishable in length, number, and structure from those seen on strain 1244. When the immunogold procedure described above was applied to 1244G7 (Fig. 2B), no reaction was seen associated with the pili; however, label was again seen associated with the O-antigen of the cell surface.

To confirm these results and to show that the strain 1244 response was not due to LPS coating of the fibers, strain 1244 pili were purified to levels in which only minor LPS contamination occurred (11). When these pili were examined by trans-

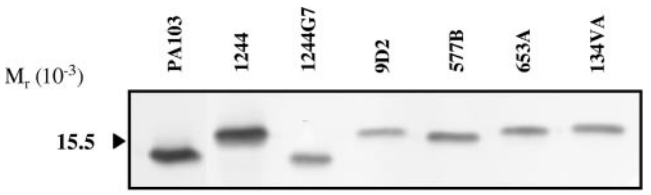

FIG. 3. Western blot of *P. aeruginosa* pilins. Cell extracts were prepared from overnight plate cultures. The blot was separated, and the PA103 lane was probed with pilin protein-specific monoclonal 2.97, while the remaining blot was probed with pilin protein-specific monoclonal 5.44.

mission electron microscopy using the immunogold protocol, it could be seen that a strong reaction still occurred and that the response was distributed evenly over the entire pilus surface (Fig. 2C). The reaction was seen only associated with the pilus. By contrast, pili purified from strain 1244G7 produced no significant immunogold reaction (Fig. 2D), although a scattered response was seen associated with minor pilus-associated debris. Outer membrane fragments contaminating these pilus preparations are the likely cause of this response. Altogether, these results indicate that the pilin glycan (i) is present at the fiber surface and (ii) is not localized to any particular region of the pilus.

Influence of pilin glycosylation on the pilus surface. The presence of the hydrophilic and negatively charged glycan on the surface of the pilus would be expected to have a significant influence on physical properties of this fiber. To test this, the relative hydrophobicities of pili from strain 1244 and the mutant 1244G7 were determined. For further comparison, pili from a strain that did not produce glycosylated pili were also examined. The apparent molecular weight of pilin from *P. aeruginosa* PA103, as determined by Western blotting using a pilin-specific serum as probe, approximated that of nonglycosylated 1244 pilin (Fig. 3), suggesting that it was not glycosylated. This was supported by MALDI-TOF analysis of pili purified from this organism (Table 2), which indicated the pilin mass to be 15,192 (±30), which agrees with the value predicted by the pilin structural gene structure (20) minus the leader sequence. In addition, purified pilin from this organism did not react with a serum specific for strain PA103 O-antigen (15), further suggesting that this protein is not glycosylated.

To test the relative hydrophobicity of the pili of strains 1244, 1244G7, and PA103, these fibers were purified and subjected to ammonium sulfate precipitation. Figure 4 shows that while the pili of strain 1244 did not come out of solution until an ammonium sulfate saturation of 45% was reached, only 15% saturation was required for precipitation of the pili from strain 1244G7. These results indicate that the presence of the glycan on the strain 1244 pilus surface resulted in a dramatic reduction of hydrophobicity. Interestingly, the pili of strain PA103, which normally are not glycosylated, were also found to be strongly hydrophobic. Purified pili from *P. aeruginosa* PAK and T2A, strains which, like PA103, produce nonmodified pili (Table 2), also came out of solution at 15% saturated ammonium sulfate (results not shown), indicating that they also presented hydrophobic surfaces.

Table 3 shows the ability of ammonium sulfate to agglutinate *P. aeruginosa* cells, an assay that determines relative surface

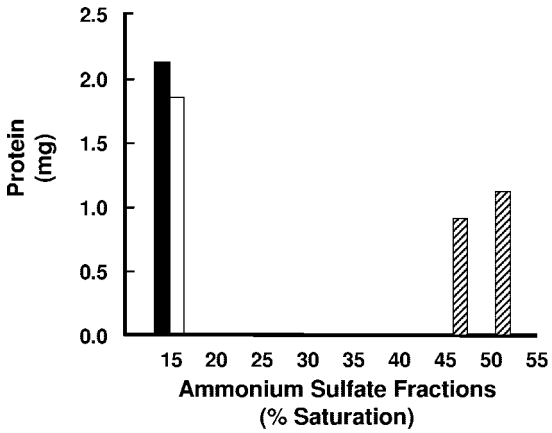

FIG. 4. Ammonium sulfate fractionation of purified *P. aeruginosa* pili. The solid bar indicates strain 1244G7 pili. The transparent bar is for pili from strain PA103. The cross-hatched bars are for strain 1244 pili.

hydrophobicity. Here it can be seen that piliated 1244 cells produced no agglutination in the presence of 15 or 25% saturated ammonium sulfate during the 25-min test period. By contrast, strain 1244G7 cells produced an agglutination response by 18 min in the presence of 15% ammonium sulfate and by 6 min in the presence of this salt at 25% saturation. Both strains were agglutinated in less than 1 min in 45% saturated ammonium sulfate. These results suggest that the glycosylation state of the pilus has an influence on the overall hydrophobicity of these cells. For comparison, 1244*wbpL*, a mutant that is unable to construct O-antigen (15), produces an agglutination response in 0.5 M ammonium sulfate within 1 min, which demonstrates the importance of this saccharide layer in modulating cell hydrophobicity. To see if the agglutination response of strain 1244G7 was due to a defect in lipopolysaccharide biosynthesis, this glycolipid was extracted from both 1244 and 1244G7 and analyzed by Western blotting using an O-antigen-specific antibody. The material extracted from strain 1244G7 appeared identical to that of wild type in amount and size distribution (results not shown), suggesting that the difference in hydrophobicity seen was not due to a variation in O-antigen composition between these strains. Altogether, these results show that the presence of the pilus glycan has a strong influence on the pilus which, in turn, influences the physical properties of the whole cell.

Influence of pilin glycosylation on pathogenicity. The results described in the previous section indicated that the presence of the glycan had a significant effect on the pilus surface; however,

TABLE 3. Ammonium sulfate-induced agglutination of *P. aeruginosa* cells

Ammonium sulfate (% saturation)	Agglutination (min)		
	Strain 1244	Strain 1244G7	Strain 1244wbpL
15	ND[a]	18	1
25	ND	6	1
45	1	1	1

[a] ND, not detectable.

TABLE 4. Frequency of group I pilins among clinical *P. aeruginosa* isolates

Source	Frequency of positives
Sputum	12/18
Urine	2/6
Ear	2/6
Wound	2/7
Unknown	5/13
Total	23/50

TABLE 5. Relative colonization by *P. aeruginosa* strains 1244 and 1244G7 in the mouse respiratory model

Animal	Cell count (CFU/g)		Ratio	Adjusted ratio[a]
	1244	1244G7		
1	4.41×10^6	1.10×10^6	4.01	2.88
2	3.20×10^7	9.90×10^6	3.23	2.32
3	1.24×10^7	2.06×10^6	6.02	4.33
4	4.24×10^7	1.23×10^7	3.45	2.48
5	5.72×10^6	1.01×10^6	5.66	4.07
6	5.83×10^6	1.60×10^6	3.64	2.62

[a] Value adjusted for ratio of strain 1244 to 1244G7 in the challenge dose.

not all *P. aeruginosa* pilins are glycosylated. These results suggest that there may be relative advantages to either arrangement and that a strain producing a particular pilus type might be more successful in occupying a specific environmental niche, including ones involved in pathogenicity. With this in mind we carried out experiments to see if pilin glycosylation had an effect on *P. aeruginosa* 1244 virulence.

The pilins of *P. aeruginosa* can be placed into at least two major groups based on primary structure and antigenicity (9). The Western blot assay presented in Fig. 3 indicates that four pilins, previously shown by sequence to belong to group I (9), all react with antibodies prepared against pure strain 1244 pilin. This suggests that this serum can be used to screen isolates for the presence of group I pilins. In addition, the apparent molecular weights of these pilins are significantly higher than that seen for nonglycosylated 1244 pilin, even though the predicted molecular weights are similar, indicating that these proteins are all posttranslationally modified. Further, testing these pilins by immunoblotting with O-specific antisera indicates that they all carry the O-antigen (results not shown). The slight variation in apparent molecular weight is likely due to differences in glycan size.

Western blot screening of whole-cell extracts of 50 clinical isolates using an anti-1244 pilin polyclonal serum showed that 23 belonged to group I (Table 4). These results were consistent with an earlier study which showed that pilin from 58 of 95 *P. aeruginosa* clinical isolates tested reacted with this antiserum as determined by Western blotting (V. J. Blanch and P. Castric, unpublished observations). The data presented in Table 4 are particularly interesting because they suggest that the group I pilins were found predominantly in strains isolated from sputum samples, suggesting the possibility that pilin glycosylation allows for enhanced pathogenicity in the respiratory tract environment.

In order to accurately test the relative virulence of strains 1244 and 1244G7, a competition assay based on the mouse acute pneumonia model was employed. Here, six mice were challenged each with a mixture of strains 1244 and 1244G7 in a ratio, as determined by growth on selective media, of 1.39:1. The challenge dose was approximately equivalent to one strain 1244 LD_{50}. These animals were sacrificed at 12 h after challenge, and the numbers of wild-type and mutant bacteria present in lung tissue were determined on the basis of growth on selective media. A competition index (12) was determined by first calculating the ratio of 1244 to 1244G7 in the lung samples (Table 5). After correcting for the ratio of these strains in the inoculum (column four of Table 5), it can be seen that strain 1244 clearly survives in the lung environment far better than does strain 1244G7. Chi-square analysis indicated that significantly more of strain 1244 than strain 1244G7 was present in the mouse lung at 12 h following challenge ($\chi^2 = 42.61$, df = 5; $P < 0.0001$). These results showed that, on average, strain 1244 was more successful in this environment by a factor of 3.12.

Since a nonspecific response to the high challenge dose used in these experiments could call into question the significance of these results, this procedure was repeated using strains 1244 and 1244G7 expressing *exoU*, a situation that substantially lowered the LD_{50} of both challenge strains. The challenge dose employed was equivalent to one LD_{50} for strain 1244 expressing the cytotoxin gene, and an initial ratio of 2.49:1 was used. The treatment of six animals produced an average competition index of 3.44, indicating that these results were consistent with the above data. Chi-square analysis ($\chi^2 = 87.9$, df = 5; $P < 0.0001$) indicated that the results were significant.

Analysis of spleen and liver tissue produced low and nonsignificant values for either strain, indicating that dissemination had not occurred at this dosage and time point. Testing an animal at time zero showed a proportion of mutant to wild type that was identical with that seen in the challenge dose. Altogether, these results indicate that the presence of the pilus glycan promotes lung colonization by *P. aeruginosa* strain 1244.

DISCUSSION

Evidence presented in this paper indicates that the pilin glycan is present at the pilus surface. Further, it is not localized but is distributed uniformly over the fiber exterior. Previous work has shown that the pilin subunit and the glycan exist in a 1:1 ratio (8). This, along with the relatively small molecular size of the glycan in comparison to the surface of the pilin subunit, suggests that this saccharide does not form a continuous layer over the pilus surface. The covalent attachment of the glycan to the carboxy-terminal pilin residue (11) places it at the edge of the subunit facet (13). This means that this structure is to be found at the seam between adjacent pilins. Altogether, these results suggest that, given the predicted subunit arrangement within the type IV pilus (14), evenly spaced glycosylation sites occur as helically arranged ascending rows of glycan on the fiber surface.

The pilus glycan organization proposed would provide ample space for interaction between the pilus protein surface and its environment. This is likely the reason why pilus-specific bacteriophages PO4 and PE69 are able to recognize glycosylated as well as nonglycosylated strain 1244 pili. Accordingly,

these viruses are also able to infect *P. aeruginosa* strain 9-D2, which produces nearly identical pilin (9) but which has a distinctively different pilin glycan (unpublished observations). These results, as well as the ability of the glycan-negative mutant to carry out twitching motility, suggest that the presence of this saccharide has a minimal effect on the ability of the pilus to extend and retract. Further, the finding that the absence of the pilin glycan in strain 1244G7 does not alter the extent of piliation suggests that the glycan does not interact with components of the pilus polymerization/depolymerization cycle.

While the pilus glycan isn't present as a continuous layer, it is capable, owing to its negative charge (8) and strong hydrophilicity, of having a significant influence on the interaction between the surface of the pilus fiber and its environment. Thus, as shown in the present work, removal of the glycan from the *P. aeruginosa* 1244 pilus greatly increases the hydrophobicity of these fibers. The evidence that pilins from strains PA103, PAK, and T2A are normally nonglycosylated and have a highly hydrophobic surface suggests that this pilus surface arrangement is not uncommon. The presence of pili with such different surface properties could facilitate the ability of particular *P. aeruginosa* strains to successfully occupy specific microenvironments. This could mean that this characteristic has a potentially important role in pathogenicity. For example, cells normally producing nonglycosylated, hydrophobic pili might more easily colonize hydrophobic environmental surfaces, such as catheters or prosthetic devices, due to increased nonspecific adherence. Here these organisms could form biofilms and subsequently disseminate to other host sites.

Strains producing glycosylated pili could interact in a different manner. The present study has provided evidence that *P. aeruginosa* group I pili are glycosylated and that strains producing them are frequently found among clinical isolates. The latter point agrees with previous studies using strain 1244 pilin-specific monoclonal antibodies (9). More importantly, group I pili-producing strains were found to be common among isolates from respiratory sources. This is consistent with the findings of Kus et al. (24), who found a high frequency of group I strains among cystic fibrosis isolates. In order to see if there is a relationship between pilin glycosylation and respiratory pathogenicity, strain 1244 and the mutant producing nonglycosylated pili were subjected to a competition assay using the mouse acute pneumonia model. This test is based on colonization of the lung and is a sensitive indicator of pathogenicity. Here, the ratio of bacteria producing glycosylated pili to those making nonglycosylated pili increased by a factor of between 3 and 4 during the initial 12-h period of challenge. These results strongly suggest that the presence of the pilin glycan makes *P. aeruginosa* 1244 a more effective respiratory pathogen than its glycan-deficient isogenic mutant. The mechanism by which pilin glycosylation might influence *P. aeruginosa* 1244 virulence in the respiratory tract is not clear; however, it is possible that this effect is due to an enhanced resistance to the nonspecific host defenses in the presence of the pilin glycan. For example, it has been shown that pili are sites of nonopsonic phagocytosis and that an increase in cell hydrophobicity is associated with killing by nonopsonic phagocytosis (43). If pilus hydrophobicity enhances this response, it is possible that the presence of the glycan on the fiber surface alters an important recognition feature, thereby preventing initial phagocyte contact. In a similar manner, the masking of portions of the pilus surface by the glycan might prevent nonopsonic complement fixation.

It is possible that the reduced lung colonization seen with strain 1244G7 was due to the modest decrease in twitching motility seen with this mutant. This seems unlikely, as previous work (12) has shown that while twitch-defective *P. aeruginosa* strains demonstrated impaired dissemination in the mouse respiratory model, there was no reduction in lung colonization.

The suggestion that pilin glycosylation in *P. aeruginosa* enhances virulence is consistent with findings that glycosylation of surface proteins of other pathogen surface proteins facilitates pathogenicity. A mannose polymer isolated from a *Chlamydia trachomatis* isolate reduced adherence by this organism, suggesting that the glycoprotein is involved in adhesion (23). *Campylobacter jejuni* mutants unable to modify proteins by means of the general glycosylation system showed reduced adherence to human and chicken cells (21). The presence of flagellin glycosylation in *P. aeruginosa* greatly increases pathogenicity as measured by the burned mouse model (2).

In addition to potentially interacting with the nonspecific host defense, the glycan might influence receptor recognition, a process that is carried out by pilin subunits located at the fiber tip (28). The 1244 pilin glycan is in close proximity to the pilin DSL ligand site (11). The saccharide may stabilize the attachment of the pilus to the host cell surface through weak bond interaction with host membrane surface components. Alternately, the glycan might function to protect the host receptor-binding site. This could be mediated by the flexibility of the oligosaccharide and by an interaction between this glycan and components of the pilin DSL. Such a situation could serve to protect the host glycolipid-binding site from proteolysis or antibody neutralization of this sensitive epitope. Studies on the structure of pilus tip subunits and on the conformation of the pilin glycan of these structures are required to clarify this point.

The location and distribution of the pilin glycan on the fiber surface make this structure a tempting target for vaccine studies. Not only do antibodies against the glycan recognize the O-antigen (11), but also an antiglycan antibody is capable of inhibiting twitching motility (8), a process which has been shown to be important in pathogenicity (12, 52). This latter response is probably due to cross-linking of the pilus fibers. It is also possible that a glycan-specific antibody acting at the pilus tip might interfere with pathogen adhesion to the host cell. Further studies should be directed towards determining if the pilin glycan represents a protective epitope and if it can be used in vaccine construction with the intended use for the protection or treatment of *P. aeruginosa* infections.

In summary, data have been presented indicating that the pilus glycan is not buried but is present at the pilus surface. In addition, this saccharide is not localized but is uniformly distributed. We have presented evidence that the pilus glycan strongly alters the pilus surface environment. While the presence of this pilin modification does not greatly influence basic pilus function, it would be expected to alter the way the pilus, and thus the whole cell, interacts with its environment, a situation that could have significance in pathogenicity. To this end, we have shown that *P. aeruginosa* strains producing glycosylated pili are commonly found among clinical isolates, espe-

cially those from the respiratory tract. Evidence was presented that the presence of the glycan enhances virulence in a mouse acute pneumonia model, suggesting that this modification predisposes strains that produce glycosylated pili to be more effective respiratory pathogens. Further experiments are required to more completely test this hypothesis.

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